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Hospital outbreak of vancomycin-resistant enterococci caused by a single clone of *Enterococcus raffinosus* and several clones of *Enterococcus faecium*

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ABSTRACT

A mixed outbreak caused by vancomycin-resistant *Enterococcus raffinosus* and *Enterococcus faecium* carrying the *vanA* gene was analysed. The outbreak occurred in a large hospital in Poland and affected 27 patients, most of whom were colonised, in three wards, including the haematology unit. The *E. raffinosus* isolates had a high-level multiresistant phenotype and were initially misidentified as *Enterococcus avium*; their unambiguous identification was provided by multilocus sequence analysis. The molecular investigation demonstrated the clonal character of the *E. raffinosus* outbreak and the polyclonal structure of the *E. faecium* isolates. All of the isolates carried the same Tn*1546*-like element containing an IS*1251*-like insertion sequence, located on a *c.* 50-kb conjugative plasmid. One of the *E. faecium* clones, found previously to be endemic in the hospital, was probably the source of the plasmid. The results of the study suggest that difficulties in identification may have led to an underestimate of the importance of *E. raffinosus* in vancomycin-resistant enterococci (VRE) control strategies.

Keywords *Enterococcus raffinosus*, identification, outbreak, Tn*1546*-like element, VRE

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INTRODUCTION

Outbreaks of vancomycin-resistant enterococci (VRE) usually occur in hospital settings, e.g., haematology wards or clinics, with severely debilitated, immunocompromised patients [1–3]. In some countries, e.g., the USA, the UK and Italy, outbreaks are relatively frequent and often result from the clonal spread of multiresistant VRE strains [2]. VRE outbreaks are mostly caused by two predominant enterococcal species, namely *Enterococcus faecalis* and *Enterococcus faecium*, whereas vancomycin-resistant organisms of other species appear only sporadically [1,2,4,5]. Thus, *Enterococcus raffinosus* is usually represented by few isolates in VRE survey studies or collections [6–11]. To our knowledge, the only description of clonal spread of vancomycin-resistant *E. raffinosus* concerned a US hospital between December 1995 and February 1996, where it affected four patients and was detected *post factum* [12]. Interestingly, the isolates of *E. raffinosus* involved were initially misidentified as *Enterococcus avium*.

Since 1997, haematology units in Poland have experienced several outbreaks of infection involving VRE, caused mostly by *E. faecium* with either VanA [13] or VanB [14,15] phenotypes. A variety of epidemiological phenomena have been identified, e.g., the parallel dissemination of several clones, horizontal transfer of *van* genes, and the multiplication and modification of *van* gene clusters, occasionally with profound consequences for the resistance phenotype. Despite the introduction of control measures, VRE still

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pose a problem in some Polish haematology units. In one such unit, with a previous history of VRE outbreaks [14], the control measures instituted included routine screening of patients for VRE carriage, combined with isolation of both colonised and infected individuals. In March 2005, a single patient appeared to be colonised by VRE with the VanA phenotype, with the responsible organism being identified by the hospital laboratory as *E. avium*, with subsequent re-identification as *E. raffinosus*. Soon after, similar isolates were recovered from 19 other patients, often accompanied by isolates of *E. faecium* that also exhibited the VanA phenotype. The aim of the present study was to characterise this new VRE-associated threat in the hospital setting.

MATERIALS AND METHODS

The VRE outbreak and associated clinical isolates

The first *Enterococcus* isolate with the VanA phenotype, which had an unusual colonial morphology on Columbia agar supplemented with sheep blood, was recovered from an asymptomatic patient in the haematology ward of the University Hospital, Kraków, Poland, on 16 March 2005. This isolate was cultured during VRE screening (rectal swabs) performed routinely every 2–3 weeks in this unit. The hospital microbiology laboratory identified the isolate as *E. avium*. After a further 3 weeks, similar isolates were obtained from a patient with urinary tract infection and from two new carriers in the haematology ward, as well as from three patients with various infections in the surgery ward. Both the colonised and the infected patients were isolated and the hospital-wide monitoring programme was intensified, resulting in further VRE identifications, including isolates from patients in the nephrology ward. By the beginning of June, 20 patients in three wards (haematology, 12 patients; surgery, six patients; nephrology, two patients) were presumed to harbour VanA$^+$ *E. avium*. In seven cases, *E. avium* was accompanied by VanA$^+$ *E. faecium*. Additionally, *E. faecium* was recovered as the only VRE from seven patients in the haematology and surgery wards. In total, 34 VRE isolates (20 *E. avium* and 14 *E. faecium*) were collected and sent to the National Medicines Institute, Warsaw, Poland, for re-identification and further analysis (Table 1). With the exception of two *E. avium* isolates, cultured from the urine and rectal swabs of the same patient, all other *E. avium* and *E. faecium* isolates were derived from different patients.

Identification of VRE isolates

The isolates were identified as enterococci according to the following criteria: Gram-positive, catalase-negative cocci; growth on bile-aesculin agar, in NaCl 6.5% w/v broth and in pH 9.6 broth; and hydrolysis of L-pyrrolydonyl-β-naphthylamide (MurexPYR; MurexDiagnostics, Dartford, UK). Identification to the species level was performed in the hospital laboratory with the RAPID32 STREP test (bioMérieux, Charbonnieres-les-Bains, France). Phenotypic identification in the National Medicines Institute was carried out with the API-20

Table 1. Selected clinical data and characteristics of the enterococcal isolates included in the study

Species[a] (no. of isolates)	Source[b]	Date of isolation (month/year); ward[c]	PFGE/ Tn*1546* hybridisation[d]	REAP/ Tn*1546* hybridisation[e]	Transfer[f]	Antimicrobial susceptibility									
						PEN	AMP	GEN	STR	VAN	TEI	CHL	TET	CIP	RIF
ER (14)	C; NI (drain, wound); I (bile, urine)	03–06/2005; H or S	a1/H1	p1–p4/h1	$+^{2,3}$	R	R	R	R	R	R	R	S, R	R	R
ER	C	04/2005; H	a2/H1	p3/h1	$+^{2}$	R	R	R	R	R	R	R	R	R	I
ER	I (bile)	05/2005; S	a3/H2	p3/h1	$+^{2}$	R	R	S	R	R	R	R	R	R	R
ER	C	04/2005; H	a4/H2	p3/h1	$+^{2}$	R	R	R	R	R	R	R	R	R	R
ER	C	05/2005; N[g]	a5/H1	p3/h1	$+^{2,3}$	R	R	R	R	R	R	R	R	R	R
ER (2)	C	05/2005; H	a6/H1	p3/h1	$+^{3}$	R	R	R	R	R	R	R	R	R	R
EFm (4)	C, NI (drain)	04/2005; H or S	A1/H1	p10, p12, p14/h1	$+^{1,2,3}$, $+^{2,3}$	R	R	R	R	R	R	S, I, R	S	R	S, I, R
EFm	C	04/2005; H	A2/H3	p10/h1	$+^{2,3}$	R	R	R	R	R	R	I	S	R	S
EFm	C	04/2005; H	A3/H1	p6/h1	$+^{3}$	R	R	R	R	R	R	I	S	R	I
EFm	C	05/2005; H	B/H1	p13/h1	$+^{1,2,3}$	R	R	S	R	R	R	I	S	R	I
EFm	C	05/2005; H	C/H1	p5/h1	$+^{1,2,3}$	R	R	R	R	R	R	R	R	R	I
EFm (2)	C; NI (wound)	05–06/2005; H	D/H1	p13/h1	$+^{2}$, $+^{2,3}$	R	R	R	R	R	R	I	R	R	R
EFm	C	05/2005; H	E/H4	p11/h1	$+^{1,2,3}$	R	R	S	R	R	R	I	S	R	S
EFm	C	05/2005; S	F/H1	p8/h1	$+^{1,2,3}$	R	R	R	R	R	R	R	R	R	S
EFm (2)	C; NI (wound)	06/2005; S	G/H5	p7, p9/h2	$+^{2}$, $+^{3}$	R	R	R	R	R	R	R	R	R	S, R

[a]ER, *Enterococcus raffinosus*; EFm, *Enterococcus faecium*.
[b]C, carriage (rectal swabs); I, infection; NI, colonisation (absence of symptoms of infection).
[c]H, haematology; N, nephrology; S, surgery.
[d]Pulsed-field gel electrophoresis (PFGE) types for *E. faecium* are designated by capital letters and those for *E. raffinosus* by lower-case letters; designations H1–H5 refer to patterns of hybridisation of the Tn*1546* probe to the *Sma*I-digested and PFGE-separated genomic DNA of the isolates (PFGE subtypes were determined for representative isolates only).
[e]REAP, restriction endonuclease analysis of plasmids; designations p1–p13 refer to *Bsp*120I restriction patterns of plasmid DNA; designations h1 and h2 refer to hybridisation patterns of the Tn*1546* probe to plasmids digested with restriction enzymes.
[f]Isolates that produced transconjugants (+) are indicated. Recipients were: [1]*Enterococcus faecalis* FA2-2; [2]*E. faecium* 64/3; [3]*E. faecium* BM4105RF. For *E. raffinosus* PFGE subtypes a1 and a6, conjugation experiments were performed for representative isolates only.
[g]One additional patient in the nephrology ward who was infected with '*E. avium*' (source, urine) was noted in 05/2005 by the epidemiological service of the hospital, but the isolate was not sent to the National Medicines Institute.
PEN, penicillin; AMP, ampicillin; GEN, gentamicin; STR, streptomycin; VAN, vancomycin; TEI, teicoplanin; CHL, chloramphenicol; TET, tetracycline; CIP, ciprofloxacin; RIF, rifampicin; R, resistant; I, intermediate; S, susceptible (for gentamicin and streptomycin, the categories refer to high concentrations of these compounds).

STREP test (bioMérieux), supplemented by tests for potassium tellurite reduction, motility and pigment production [16], and with the AST-P534 panel card in the VITEK 2 Compact Version system (bioMérieux, Durham, NC, USA). DNA-based species identification was performed using multilocus sequence analysis (MLSA) of the *rpoA* and *pheS* genes, as described by Naser *et al*. [17]. *E. avium* ATCC 14025, *E. faecalis* ATCC 29212, *E. faecium* BM4147 [18] and *Enterococcus gallinarum* BM4174 [19] were used as control strains.

Antimicrobial susceptibility testing

MICs of antimicrobial agents were determined by the agar dilution method according to CLSI guidelines [20]. The following agents were tested: penicillin, ampicillin, gentamicin, streptomycin, tetracycline, rifampicin and chloramphenicol (Polfa Tarchomin, Warsaw, Poland); vancomycin (Lilly, Indianapolis, IN, USA); teicoplanin (Marion Merrell Dow, Denham, UK); and ciprofloxacin (Bayer, Wuppertal, Germany). Susceptibility to linezolid was tested using Etests (AB Biodisk, Solna, Sweden). *E. faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, *E. faecium* BM4147 (VanA) and *E. faecium* BM4147-1 (vancomycin-susceptible) reference strains [18] were used as controls.

Resistance transfer

Transfer of vancomycin resistance was investigated using the filter-mating procedure, with *E. faecalis* FA2-2, *E. faecium* 64/3 and *E. faecium* BM4105RF (resistant to rifampicin and fusidic acid) as recipients [21]. Transconjugants were selected on brain–heart infusion agar (Oxoid, Basingstoke, UK) containing rifampicin 64 mg/L, fusidic acid 64 mg/L (Leo Pharmaceutical Products, Ballerup, Denmark) and vancomycin 32 mg/L in the case of donor isolates with rifampicin MICs ≤8 mg/L, and on agar containing fusidic acid and vancomycin in the case of donors with rifampicin MICs ≥32 mg/L. Conjugation was verified by comparative pulsed-field gel electrophoresis (PFGE) analysis of transconjugants against donor and recipient strains, and by detection of the *vanA* gene by PCR in the transconjugants (see below).

PFGE typing

Genomic DNA of the isolates, embedded in agarose 0.75% w/v plugs (InCert Agarose; FMC Bioproducts, Rockland, ME, USA), was digested with *Sma*I (Fermentas, Vilnius, Lithuania) and separated in Pulsed Field-Certified agarose (Bio-Rad, Hercules, CA, USA) in a CHEF DR III system (Bio-Rad). DNA was purified as described by Clark *et al*. [6]. PFGE was performed using the same run conditions for both species (voltage 6 V; pulse time 1–25 s; run time 22 h). PFGE patterns were analysed according to the criteria of Tenover *et al*. [22]. *E. avium* ATCC 14025 and *E. faecium* BM4147 [18] were used as control strains.

Detection of the *vanA* gene

Total DNA was purified from the isolates using the Genomic DNA Prep Plus kit (A&A Biotechnology, Gdańsk, Poland). The *vanA* gene was detected using specific PCR primers as described by Clark *et al*. [6]. DNA isolated from *E. faecium* BM4147 [18] was used as a positive control.

Amplification of Tn*1546*-like transposons and *vanRSHAX* regions by long-PCR (L-PCR) and subsequent restriction fragment length polymorphism analysis

Tn*1546*-like elements were amplified from total DNA of the isolates using the Expand Long Template PCR System (Roche Diagnostics, Mannheim, Germany). Subsequently, these amplicons were used as templates in L-PCR of the *vanRSHAX* regions. Both PCRs were performed as described by Palepou *et al*. [23]. The L-PCR products containing Tn*1546*-like elements were digested with *Bsu*15I (an isoschizomer of *Cla*I; Fermentas) and the *vanRSHAX* amplicons were digested with *Dde*I (Promega, Madison, WI, USA) [23]. DNA from *E. faecium* BM4147 [18] was used as a control.

Sequencing of the *vanS–vanH* intergenic region

The *vanS–vanH* intergenic region was amplified by PCR using a forward primer annealing to the *vanS* gene (5′-AATTATTGTTCAGCATGGAGGGCAG) and a reverse primer annealing to the *vanH* gene (5′-TTTGGCGTTGGATTCCGACAC). PCR products were purified with the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and were then partially sequenced using the same primers, and an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA, USA).

Genetic location of Tn*1546*

The *Sma*I-digested and the undigested total DNA of the isolates were separated by PFGE (as described above), blotted on Hybond-N membranes (Amersham Pharmacia Biotech, Little Chalfont, UK), and hybridised with the Tn*1546* probe, using the ECL Random-Prime labelling and detection system (Amersham Pharmacia Biotech). The probe was obtained by labelling the L-PCR amplicon of the entire Tn*1546* element from *E. faecium* BM4147 [18]. Total DNA of *E. faecium* BM4147 [18] was used as a positive control in the hybridisation experiment. Plasmid DNA was purified from bacterial spheroplasts using a Plasmid Midi Kit (Qiagen) as described previously [15]. DNA preparations were digested separately with *Bsp*120I, *Eco*RI and *Bsu*15I (Fermentas), electrophoresed in agarose 1% w/v gels, blotted and then hybridised with the Tn*1546* probe.

RESULTS

Species identification

The 20 enterococcal isolates classified as *E. avium* by the hospital laboratory were subjected to several identification procedures. The classical protocol utilising the API-20 STREP test (bioMérieux) and the scheme of Facklam and Collins [16] confirmed the initial identification as *E. avium*. However, fundamental differences in the PFGE patterns of the isolates, when compared with the *E. avium* ATCC 14025 reference strain (Fig. 1) [12], and the resistance of the

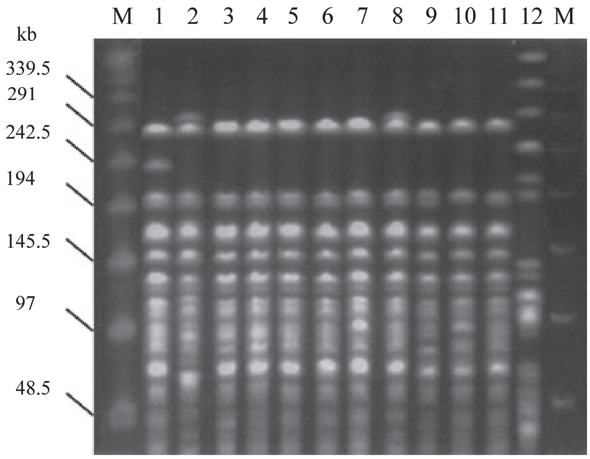

Fig. 1. *Sma*I pulsed-field gel electrophoresis patterns of selected *Enterococcus raffinosus* isolates. Lanes: M, λ DNA Ladder (New England Biolabs, Beverly, MA, USA); 1, isolate 2229/05 (PFGE subtype a2); 2, isolate 2225/05 (a3); 3, isolate 2226/05 (a1); 4, isolate 2223/05 (a1); 5, isolate 2209/05 (a1); 6, isolate 2215/05 (a1); 7, isolate 2217/05 (a6); 8, isolate 2205/05 (a4); 9, isolate 2229/05 (a5); 10, isolate 2220/05 (a6); 11, isolate 2208/05 (a1); and 12, *Enterococcus avium* ATCC 14025.

isolates to penicillins, which is uncommon in *E. avium* [24], prompted a re-identification. The VITEK 2 Compact Version system identified the isolates as *E. raffinosus* with a rank of 'excellent' or 'very good' according to the expert programme. Unambiguous identification was achieved with the MLSA approach [17] using a representative isolate, which revealed 100% identity with the *pheS* and *rpoA* genes of *E. raffinosus*. In the case of the 14 *E. faecium* isolates, identification by the classical procedure and the VITEK 2 system was unequivocal.

Antimicrobial susceptibility testing

The susceptibility categories are presented in Table 1. All of the *E. raffinosus* and *E. faecium* isolates were resistant to penicillin and ampicillin (MICs 16 - >128 mg/L), glycopeptides (vancomycin MICs 128–1024 mg/L; teicoplanin MICs 32–128 mg/L) and ciprofloxacin (MICs 16 - >64 mg/L). High-level resistance was uniform in the case of streptomycin (MICs ≥2048 mg/L), whereas one isolate of *E. raffinosus* and two of *E. faecium* remained susceptible to high concentrations of gentamicin (MICs 8–16 mg/L). Susceptibility to tetracycline and rifampicin differentiated the two species, with almost all of the *E. raffinosus* isolates being non-susceptible and the majority of *E. faecium* isolates being susceptible or intermediately-susceptible to these compounds. All isolates were susceptible to linezolid.

PFGE typing

The results of the PFGE analysis are presented in Table 1. Fig. 1 shows the PFGE patterns of 11 selected *E. raffinosus* isolates. All the *E. raffinosus* isolates belonged to a single type, designated 'a', which was divided into several subtypes (a1–a6) [22]. The most common of these was PFGE subtype a1 (15 isolates), which was identified in isolates from the haematology and surgery wards. In contrast, seven PFGE types (A–G) were distinguished among the *E. faecium* isolates, with the predominant type A represented by six isolates from the haematology and surgery wards. These isolates could be differentiated into subtypes A1–A3. Two indistinguishable *E. faecium* isolates from the haematology ward were classified into type D, and two isolates from the surgery ward into type G. The remaining types were unique.

Detection of the *vanA* gene

A fragment of *c.* 1030 bp, corresponding to the expected size of the *vanA* gene, was amplified from all of the enterococcal isolates (data not shown).

Resistance transfer

Each of the six *E. raffinosus* isolates tested, representing each of the PFGE subtypes, as well as all the *E. faecium* isolates, transferred the glycopeptide resistance determinants to at least one of the recipient strains (Table 1). The *vanA* gene was detected in all the transconjugants obtained, which had a pulsotype similar to the respective recipients and distinguishable from the donor isolates (data not shown). In general, the frequency of transfer observed for *E. raffinosus* was three to five orders of magnitude lower than that observed for *E. faecium*. Five *E. faecium* isolates, but none of the *E. raffinosus* isolates, yielded transconjugants with *E. faecalis* FA2-2.

Amplification of Tn*1546*-like transposons and *vanRSHAX* regions by L-PCR and subsequent restriction fragment length polymorphism analysis

The Tn*1546*-like transposon harbouring the *vanA* gene was amplified from all *E. raffinosus* and *E. faecium* isolates, although with remarkably lower efficiency than from the *E. faecium* BM4147 control strain (data not shown). Restriction analysis with *Bsu*15I (*Cla*I) revealed a uniform DNA pattern (Fig. 2), which included two bands of *c.* 2.5 and 2.0 kb that were common to the original Tn*1546* variant from BM4147 [18], and an additional band of *c.* 4.5 kb and a double (or triple) band of *c.* 500 bp.

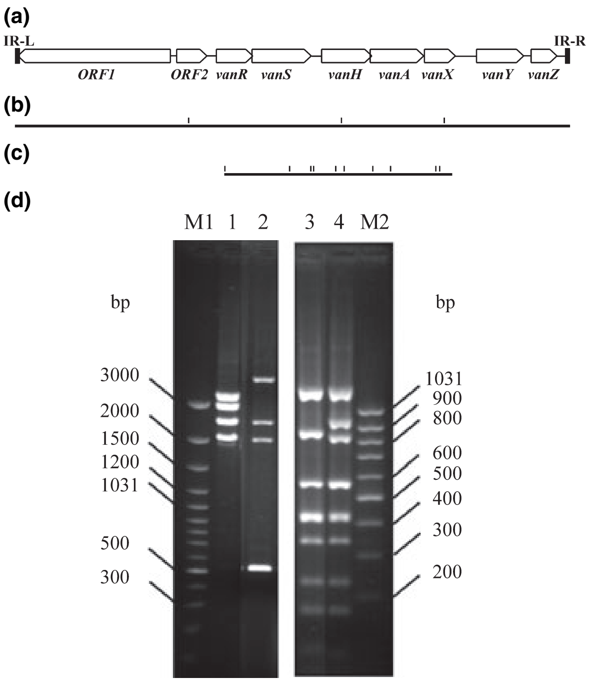

Fig. 2. (a) Schematic representation of the structure of transposon Tn*1546* from *Enterococcus faecium* BM4147 [18]. (b) The long-PCR (L-PCR) amplicon of Tn*1546*, obtained as proposed by Palepou *et al.* [23], used in the restriction fragment length polymorphism (RFLP) analysis with the *Bsu*15I (*Cla*I) restriction enzyme and as a probe in hybridisation studies; the distribution of *Cla*I restriction sites is indicated by vertical strokes. (c) The L-PCR amplicon of the *vanRSHAX* region, obtained according to Palepou *et al.* [23], used in the RFLP analysis with *Dde*I (location of *Dde*I restriction sites is indicated). (d) *Bsu*15I (*Cla*I) polymorph VI of the Tn*1546*-like transposon and *Dde*I polymorph II of the *vanRSHAX* gene cluster. Lanes: M1, Gene Ruler 100-bp DNA Ladder Plus (Fermentas, Vilnius, Lithuania); 1, *Bsu*15I (*Cla*I) digest of Tn*1546* of *E. faecium* BM4147 [18]; 2, *Bsu*15I (*Cla*I) polymorph VI of the Tn*1546*-like transposon; 3, *Dde*I digest of the *vanRSHAX* gene cluster of *E. faecium* BM4147 [18]; 4, *Dde*I polymorph II of the *vanRSHAX* gene cluster; and M2, Gene Ruler 100-bp DNA Ladder (Fermentas).

The *vanRSHAX* gene cluster was amplified from each of the outbreak isolates, and was found to be significantly larger in size than that present in BM4147 [18], indicating that a DNA insertion must have occurred inside the cluster.

Sequence of the *vanS–vanH* intergenic region

Differences in restriction patterns between the *vanRSHAX* polymorph and *E. faecium* BM4147 [18] suggested that the DNA insertion had probably occurred in the *vanS–vanH* intergenic region. This region was amplified from representative isolates of *E. raffinosus* and *E. faecium*, and was then subjected to partial sequence analysis. The size of the PCR products indicated that the insertion was *c.* 1.5 kb in size, and DNA sequencing revealed the presence of an IS*1251*-like element (*c.* 500 bp from the *vanS* side and *c.* 100 bp from the *vanH* side). The element was located at the same position and in the same orientation as that found in Tn*1546*-like transposons from the USA [25], Norway and Ireland [26] and Brazil [27], and the sequence fragments were identical to those described by Simonsen *et al.* [26] (GenBank accession number AF148130) and Camargo *et al.* [27] (AY560917).

Genetic location of Tn*1546*

The Tn*1546* probe was used to detect glycopeptide resistance determinants in *Sma*I-digested genomic DNA of representative isolates. Among seven *E. raffinosus* isolates analysed, there were two different PFGE hybridisation patterns, H1 and H2 (Table 1), each consisting of a single DNA band migrating between the 48.5-kb and 97-kb bands of the λ ladder DNA molecular size marker. For almost all of the *E. raffinosus* PFGE subtypes, the H1-specific band migrated slightly slower than that of the H2 pattern, which was observed in an isolate with PFGE subtype a3. The hybridising bands were in non-equimolar proportions to other bands, which suggested a possible plasmid association. This hypothesis was confirmed by PFGE analysis of undigested total DNA from representative isolates belonging to PFGE subtypes a1 and a2. The hybridisation signals were identified at the same positions in the gel as were observed with *Sma*I-digested DNA, which indicated the absence of an *Sma*I restriction site in the plasmid(s) carrying the *vanA* cluster (results not shown).

The H1 pattern of hybridisation was most frequent among the nine *E. faecium* isolates analysed, which represented all of the PFGE types and subtypes identified in the study (Table 1). Some isolates were characterised by single bands of other sizes (PFGE subtype A2, pattern H3; type E, pattern H4). The isolate belonging to PFGE type G yielded a specific pattern, designated H5, with three hybridising bands, one between 194 kb and 242.5 kb, one just above 242.5 kb, and one below the 339.5 kb band of the λ DNA ladder (results not shown).

Characterisation of plasmids carrying Tn*1546*

Plasmid DNA from each of the outbreak isolates was purified and digested with *Bsp*120I. Highly diverse restriction patterns were observed, four in *E. raffinosus* isolates and ten in *E. faecium* isolates (Table 1), indicating the presence of multiple plasmids in various combinations in particular groups of the isolates. However, when these patterns were hybridised with the Tn*1546* probe, the result was almost uniform, showing one band of high molecular size (>10 kb), designated pattern h1 (Table 1). Only two *E. faecium* isolates (PFGE type G) yielded a smaller band (pattern h2). The study was continued using plasmid DNA from the isolates and their transconjugants, which was digested with *Bsu*15I (*Cla*I) and *Eco*RI separately. The results obtained were similar, with both enzymes yielding sets of identical hybridisation patterns for all but the two *E. faecium* isolates of PFGE type G (results not shown).

As shown in Fig. 3, the transconjugant of *E. faecium* isolate 2227/05 most probably harboured a single plasmid molecule of *c.* 50 kb, which yielded 16 bands following digestion with *Eco*RI (the plasmid size was calculated by calibrating the size of the *Eco*RI restriction fragments). DNA hybridisation revealed that three of these bands encompassed the Tn*1546*-like element.

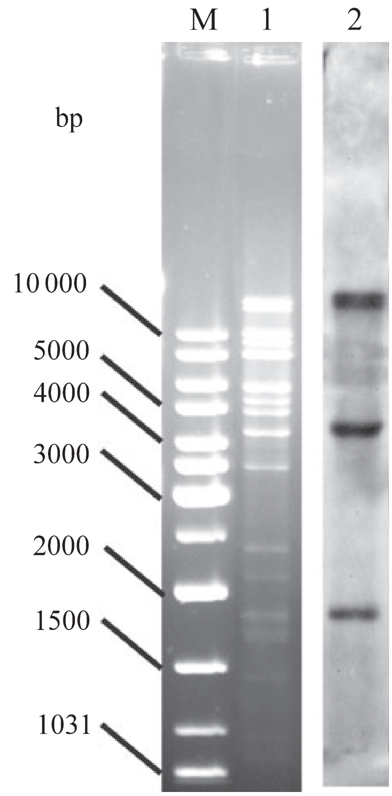

Fig. 3. *Eco*RI restriction pattern of the *c.* 50-kb plasmid carrying the Tn*1546*-like element, showing the hybridisation pattern with the Tn*1546* probe. Lanes: M, Gene Ruler DNA Ladder Mix (Fermentas, Vilnius, Lithuania); 1, plasmid DNA isolated from a transconjugant of *Enterococcus faecium* 2227/05 digested with *Eco*RI (plasmid *c.* 50 kb); and 2, hybridisation of the Tn*1546* probe to the fragments of the *c.* 50-kb plasmid shown in lane 1.

DISCUSSION

The aim of this work was to investigate the genetic background of a VRE outbreak in a large medical centre in Poland, in which outbreaks of *E. faecium* or *E. faecalis* strains with the VanA or VanB phenotypes have already been reported [14]. The number of patients affected (n = 27) was remarkably high, but most of the VRE isolates were recovered from the faeces of colonised patients. One of the most distinctive aspects of the outbreak was the predominance of a single *E. raffinosus* clone. Using classical methods, isolates of this clone were initially identified as *E. avium*; however, the VITEK 2 system and the MLSA molecular approach [17] both classified the isolates unequivocally as *E. raffinosus*. Since *E. avium* has been associated more often with human infection, and has been reported to be vancomycin-resistant more frequently than has *E. raffinosus* [28–33], there was a possibility of misidentification of these two species. Wilke *et al.* [12], reported that six of eight cases of bacteraemia identified by the VITEK system as being caused by *E. avium* were actually caused by *E. raffinosus*. Interestingly, the re-identification made use of conventional methods [16], which, in the context of the present experience, underlines the significance of MLSA. As mentioned previously, the study by Wilke *et al.* [12] is probably the only report of VanA$^+$ *E. raffinosus* clonal spread to date; however, since these isolates were collected as part of a surveillance programme [7], the description was fragmentary [12].

The results obtained in the present study indicate the occurrence of an outbreak caused by a single strain of *E. raffinosus*, in which all isolates belonged to a single PFGE type with almost uniform resistance patterns and levels. Although a polyclonal population of *E. faecium* isolates was observed, pulsotype A was predominant. The same Tn*1546*-like transposon was observed in the other enterococcal isolates studied, suggesting that dissemination among different species and clones had occurred.

The Tn*1546*-like transposon contained the IS*1251*-like element inside the *vanS–vanH* intergenic region. Comparative restriction analysis with the original Tn*1546* element [18] also indicated rearrangements in the 5′ region (*orf1* and *orf2*), although these were not essential in resistance expression. The positive L-PCR with a primer that targeted the Tn*1546* inverted repeats (IRs) [23] demonstrated that this polymorph did indeed contain the IRs; however, the low efficiency of the L-PCR suggested that these (most probably IR-L) were also distorted. It is very likely that this polymorph is related, or even identical, to the IS*1251*-containing Tn*1546*-like elements originally found in the USA [25] and later observed in other countries [26,27], which thereby provides evidence for their further dissemination. It should be emphasised that instability of Tn*1546*-like transposons has often been reported and attributed to changes within the *orf1–orf2* region and/or the presence of IS elements [23,25,26,34–37].

The present hypothesis that horizontal spread of the Tn*1546*-like transposon among clones had occurred was supported by hybridisation of Tn*1546* to several putative plasmids. One of these was predominant (hybridisation pattern H1) and was further characterised as a conjugative molecule of *c.* 50 kb. It is possible that plasmids of other sizes (patterns H2–H4) were derivatives of H1, as indicated by the uniform hybridisation of Tn*1546* with plasmids H1–H4 that had been purified and cut with restriction enzymes (hybridisation pattern h1). Two *E. faecium* isolates belonging to PFGE type G differed significantly in terms of the location of the Tn*1546*-like element, which was found to reside in a different plasmid context (pattern H5), either in a single molecule observed in three conformations according to PFGE, or in two or three different plasmids. The H5-specific plasmid(s) might have acquired the Tn*1546*-like element from H1; indeed, one of the *E. faecium* isolates was recovered from the same site (wound) as one of the *E. raffinosus* isolates. Previous studies have shown that the content and structure of plasmids in enterococcal strains can change quickly, even during ongoing clonal outbreaks [13–15].

Most probably, the *E. faecium* clone defined as PFGE type A acted as a reservoir for the glycopeptide resistance determinant found in the isolates in this study. During 2002–2003, this clone had been responsible for a successfully controlled outbreak in the haematology ward. However, even if clone A had not been isolated from new haematology patients during 2004, it could have persisted in other wards that were not covered by the strict VRE control system. The first *E. faecium* isolate belonging to PFGE type A was identified during the new outbreak at almost the same time as the first *E. raffinosus* isolate, and clone A predominated among *E. faecium* isolates.

The outbreak described in this study occurred despite the extensive efforts of the staff of a medical centre experienced in controlling VRE. As with similar outbreaks reported to date, the outbreak had a very complex genetic background. This study has clearly demonstrated that some rarely encountered *Enterococcus* spp., which often fail to be identified by widely used identification systems, may become important reservoirs of *van* genes and contribute significantly to the dissemination of resistance. Accordingly, certain widely held beliefs concerning these organisms, including *E. raffinosus*, e.g., that 'the majority of isolates do not transmit vancomycin resistance genes or proliferate' [38], may come into question in the near future, and *E. raffinosus* should be taken into consideration when devising control procedures for enterococcal infections.

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